REVIEW ARTICLE

Insulin receptors in breast cancer: Biological and clinical role

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INTRODUCTION

Breast cancer is a common malignancy that affects almost 1 in every 7 women and it is the leading cause of death in women of many Western countries (1). Although advances in the molecular biology have allowed to evidence many molecular alterations in breast cancer (2, 3), it is not well understood which mechanisms are primarily relevant in leading normal cells to neoplastic transformation. Recent observations suggest that hereditary factors account only for approximately 15% of breast cancer in humans (4). Therefore, hormones, dietary habits and other environmental factors play a major role in approximately 85% of breast carcinomas.

As far as prognosis is concerned, so far the main prognostic index is the presence of nodal metastases (5). However, since approximately 50% of node negative carcinomas will relapse, a series of cancer cell abnormalities (e.g. erbB2 amplification, overexpression of EGF receptors, alteration of p53 antionogene, etc.) have been proposed to identify those node negative carcinomas which are likely to recur and should, therefore, be treated with adjuvant chemotherapy. The clinical relevance of these molecular alterations is however not well established.

This paper reviews evidences indicating that: a) hyperinsulinemia and overexpression of insulin receptor (IR) play a role in breast cancer; b) measurement of IR in breast cancer specimens is helpful in evaluating prognosis in patients with node negative carcinomas.

These findings may open new possibilities in breast cancer prevention, prognosis assessment and therapy.

HORMONES, GROWTH FACTORS AND BREAST CANCER

It is now well accepted that growth factors involved in the normal cell growth and differentiation may be also involved in the neoplastic transformation. In fact, alterations in the availability of growth factors, in the expression of their receptors or in their intracellular signaling pathways may favor or determine cell transformation (6).

Cell transformation is caused by a critical number of mutations in genes (e.g. oncogenes and tumor suppressor genes) involved in cell proliferation and differentiation. A certain level of endogenous DNA damage is always present in the cell genome and it is increased by the exposure to mutagens (7). The cells, however, possess mechanisms able to repair damaged DNA or to stop proliferation in cells with damaged DNA in order to prevent accumulation of DNA mutations. Growth factors and hormones able to stimulate cell proliferation reduce the effectiveness of the cell DNA repairing machinery and, therefore, increase the rate of DNA mutations and favor cell transformation. The simultaneous exposure to mutagens and growth factors increases the possibility of cell transformation.

An extensive literature has addressed the role of estrogens and progestagens in the development and in the prognosis and therapy of breast cancer (8, 9). It is now well established that factors which increase estrogen and progesterone exposure (e.g. early menarche, delayed menopause, prolonged estrogen therapy in postmenopause) also increase the risk of breast cancer.

However, not only steroids but also a variety of polypeptide hormones and growth factors play an important role in normal breast development and are possibly involved also in breast cancerogenesis. Epidermal growth factor (EGF), insulin, insulin-like growth factor-I (IGF-I), GH, PRL, are all important in the growth and development of different parts of the mammary epithelial tree (ducts, end buds, alveoli) (Figure 1). The possible role of growth factors of the EGF family in breast cancer has been...
addressed by numerous studies and has been reviewed elsewhere (10).
Despite early evidences obtained in the animal model that insulin affects the growth of breast cancer, the possible role of insulin and insulin-like growth factors in this cancer has been less studied. Insulin and insulin-like growth factor I (IGF-I) are closely related peptides (11). However, while insulin is commonly believed to exert mainly metabolic effects, IGF-I is considered a major factor for the regulation of cell proliferation (12, 13).

INSULIN AND BREAST CANCER
a) Animal models
Twenty-five years ago Heuson et al. (14) reported that breast tumors induced by 7,12-dimethylbenz(a)anthracene (DMBA) in the rat were induced to regress when the rats were made diabetic by alloxan administration. Administration of insulin but not of estrogens caused resumed tumor growth. Similar result were obtained by Cohen and Holll (15) who used streptozotocin to induce diabetes. These tumors contained specific receptors for insulin which were found to have binding characteristics similar to those present in normal cells. Interestingly, DMBA-induced tumors which continued to growth after ovariectomy showed increased insulin binding as compared to tumors which regressed after ovariectomy. Since insulin enhances glucose uptake and enhanced glucose availability may be important for neoplastic cell growth, Heuson and Legros studied the role of insulin and of glucose in thymidine incorporation into DMBA-induced tumor explants. They concluded that the insulin-induced stimulation of thymidine incorporation was not the simple consequence of enhanced glucose uptake although glucose was rate-limiting (16).
Recently, Dryer et al. (17) have reported that treatment of female rats with an insulin analog (AspB10) which have an increased affinity for IR, had carcinogenic effect on mammary gland. They found, at the end of a 24-month period, that 44% mice developed benign breast diseases and 23% developed breast cancer. Conversely, Fernandes et al. (18) have found that lipids and calorie restriction significantly inhibit the development of mammary tumors in mouse mammary tumor virus/m-Ha-ras transgenic mice. Although circulating insulin levels were not measured it is likely that they were reduced by caloric restriction.
Of considerable interest is a different model of mammary carcinoma of the rat, the transplantable R3230AC carcinoma (19). This carcinoma has several properties of lactating breast tissue including the presence of estrogen receptors and secretion of casein. In contrast to DMBA-induced tumors, R3230AC carcinoma grows faster in diabetic animals and its growth is inhibited by insulin or estrogen administration (20). These data suggest that insulin, like estrogens, can have a dual action on breast cancer. This is in agreement with the known effects of insulin on cell differentiation.
b) Human studies
Obesity and epidemiology of breast cancer
A number of studies have addressed the possibility that overweight may be associated with advanced breast cancer. Given the modifying nature of this risk factor, these studies are of interest in breast cancer prevention. Indeed, overweight has been found to be associated with increased risk of developing breast cancer, with a more advanced disease and positive axillary nodes at diagnosis and with early cancer recurrence (21, 23). Some studies, however, have not confirmed the association with these increased risk in premenopausal patients. However, most authors agree that the risk of developing breast cancer is associated directly with body mass index measured at later adult ages (22). Particularly interesting are studies taking into account the distribution of body fat. Abdominal adiposity (measured as waist-to-hip ratio) has been found positively associated with increased risk of pre- and/or postmenopausal breast cancer (24).

Although the mechanism of this association has not been worked out, these findings have been generally interpreted in the framework of the estrogen etiologic hypothesis. Obese women generally have higher concentrations of estradiol, non-protein-bound estradiol and estrone which is produced from aromatization of androstenedione in the fat tissue (25, 26). This increased estrogen availability would have little effect in premenopausal women but would increase breast cancer risk in postmenopausal women. In our opinion this explanation is not satisfactory. Abdominal obesity (android type) in postmenopausal women is not associated with evidence of increased estrogen effects; in fact these women have androgen excess (26), and are not protected from but actually at high risk for cardiovascular disease. A more convincing hypothesis is that hyperinsulinemia is partially responsible for this association between breast cancer and abdominal adiposity. In fact, abdominal adiposity is strongly associated with insulin resistance which determines higher than normal fasting and postprandial circulating insulin levels (27, 28). In concert with our hypothesis is the recent finding of Barnes-Josiah et al. (29) that weight gain after age of 18 is associated with significant increase in breast cancer development. Recent studies have provided evidence that even a small weight gain after age of 18 is associated with insulin resistance and increased risk of NIDDM. Moreover, Bruning et al. (30) have found a positive association between insulin resistance and breast cancer.

Diabetes and epidemiology of breast cancer
Although some studies have found a higher incidence of diabetes in patients with breast cancer as compared with patients with benign breast diseases or with the general population, results have not been univocal (20). Further studies would take into account the type of diabetes (insulin dependent vs. non insulin dependent diabetes).

INSULIN RECEPTORS IN HUMAN BREAST CANCER
Although a number of evidences individuate a major role of insulin in the biology of mammary epithelial cells, up to ten years ago very little information was available on insulin receptor expression and function in breast cancer tissues. The insulin receptor (IR) is a tetrameric glycoprotein which belongs to a family of transmembrane growth factor receptors with tyrosine kinase activity (13, 31). IR has more than 50% amino acid sequence homology with the IGF-I receptor (IGF-I-R). The highest degree of sequence homology (84%) is seen in the tyrosine kinase domain. It is not yet entirely clear whether the different (metabolic vs. mitogenic) ascribed to insulin and IGF-I, respectively, are the results of differences in intracellular signaling between these two closely related receptors or to other mechanisms, e.g. different expression level of the 2 receptors in different tissues. Available studies comparing the intracellular pathways of the 2 receptors have shown only subtle or no differences (12, 13).

1- Insulin receptor overexpression in human breast cancer

a) Studies in breast cancer specimens:
We measured IR expression in a series of 308 human breast cancer specimens by using a specific and sensitive radioligand assay (32) and compared it to the values found in both benign fibroadenomas (n=9) and in normal breast tissue specimens (n=42) (Figure 2).

In breast cancer specimens the average IR content was 8.6±4.4 ng/0.1 mg protein, significantly higher (p<0.001) than values found in both benign fibroadenomas (1.1±0.5) and normal breast tissues (1.0±0.7 ng/0.1 mg protein). In over 80% of cancers the IR protein content was higher than the mean value + 2SD found in normal breast tissue. Values over 10 fold higher than mean value in normal tissue were observed in approximately 20% of breast cancer specimens (33). Immunostaining indicated that IRs were actually overexpressed by the neoplastic epithelial cells. Myoepithelial and endothelial cells were
also stained with little or no staining in stromal adipocytes and inflammatory cells.

b) Potential prognostic value of IR measurement

To investigate the potential prognostic value of IR measurement in breast cancer we evaluated IR expression in a retrospective series of 531 node-negative breast carcinomas by immunohistochemistry. We found that patients with tumors having both high and undetectable IR content had a shorter disease-free interval. The prognostic value of IR measurement was greater than that of other established markers including estrogen receptor (ER), progesterone receptor (PgR), S-phase and ploidy. In patients with ER-tumors, IR measurement predicted both a shorter disease-free interval and overall survival (unpublished data). If prospective studies will confirm these findings IR measurement may become a tool able to identify patients with node-negative breast cancers who would benefit from adjuvant chemotherapy.

c) Studies in human breast cultured cell lines:

Human breast cells in tissue culture represent important in vitro models for studying the regulation of breast cancer tissue by hormones and growth factors. The presence of insulin binding sites in human breast cancer cells in culture has been reported almost 20 years ago (34). We directly measured insulin receptor content by a specific radiomunooassay in extracts of several breast cancer cell lines and of the non malignant human breast cell line, MCF-10 (35). IR were overexpressed in most of breast cancer cell lines (Table 1). As in human breast cancer specimens, IR expression in human breast cancer cultured cells varied within a wide range, being 0.5-20 fold in comparison to the non malignant human breast cells (36).

IGF-I-Rs were also measured in both breast cancer tissues and cell lines by radiomunooassay (37). Interestingly, in breast cancer specimens mean IR content was similar to the mean IGF-I-R content. In contrast, IGF-I-Rs were expressed at a higher level than IRs in cultured breast cancer cells: this finding could be due to adaptation to the in vitro conditions and suggests that insulin and IGF-I are equally important in vivo (Table 1).

d) Mechanisms of insulin receptor overexpression

The mechanism responsible for elevated IRs can include gene amplification accompanied by rearrangements that produce altered transcripts, gene amplification without rearrangement, and overexpression of IR in the absence of gene amplification. Human cell lines with a non-rearranged IR gene contain several species of IR mRNA (ranging from 11.6 to 5.2 kb), due to variable splicing at the 3' end of the IR RNA. There are also multiple 5' start sites, but they differ by only several hundred of bases and thus cannot account for such size heterogeneity (38). Most likely, all these multiple mRNA species correspond to a full-length cDNA and are involved in IR protein synthesis. In all breast cancer cells and specimens examined, two prominent bands of IR mRNA were seen at 11.0 and 8.5 kb, although the ratio between the two bands was very

<table>
<thead>
<tr>
<th>Breast cell lines</th>
<th>IR RIA (ng/10^6 cells)</th>
<th>IGF-IR RIA (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td>28.5±4.5</td>
<td>110.0±15.0</td>
</tr>
<tr>
<td>MDA MB 231</td>
<td>29.1±5.4</td>
<td>8.0±1.2</td>
</tr>
<tr>
<td>ZR 75.1</td>
<td>17.1±1.9</td>
<td>18.0±2.0</td>
</tr>
<tr>
<td>T47 D</td>
<td>4.8±0.8</td>
<td>48.0±6.0</td>
</tr>
<tr>
<td>MCF-10</td>
<td>5.6±1.1</td>
<td>4.6±1.0</td>
</tr>
</tbody>
</table>

Table 1 - insulin receptor (IR) and insulin-like growth factor-I receptor (IGF-IR) content in breast cell line in culture evaluated by radiomunooassay. Data are mean ± SEM of 4 determinations.
variable. No major quantitative differences were observed in the specific IR mRNA and the level of these transcripts correlates with the amount of IR protein, suggesting that the observed difference in IR protein expression are controlled to a great extent at the transcriptional level (36).

Analysis of the IR gene indicate that amplification in breast cancer tissues is an uncommon event in respect to the high prevalence of IR protein overexpression. In our series of 96 primary breast carcinomas studied by Southern blot and fluorescence in situ hybridization (FISH) an increased IR gene copy number was observed in only 8% of primary breast carcinomas and in 1/6 breast cancer cell lines (MDA-MB231 cells) (unpublished data).

II- Insulin receptor function in breast cancer

In both tissue specimens and cultured cell lines we investigated IR function in terms of ligand binding ability and tyrosine kinase capacity. Since early studies have not clarified whether insulin induces proliferation in breast cancer cells through IRs or via cross reaction with the related IGF-I-Rs we also studied the ability of IRs to mediate growth stimulation.

a) Tissue specimens

The structure of the α-subunit of the breast cancer insulin receptor was investigated by cross-linking with 125I-insulin followed by SDS-PAGE and the functional capacity by 125I-insulin binding studies. Results suggested no major differences between IRs in cancer and normal breast tissues (39). The tyrosine kinase activity of the IR β-subunit was then studied by measuring IR autophosphorylation and phosphotransferase activity in response to insulin. Basal and maximal insulin-stimulated (100 nM) IR autophosphorylation was similar in both cancer and normal tissues; however, low insulin concentrations (3 nM) caused 69.5% of maximal IR activation in breast cancer tissue vs. 42.5% in normal breast tissue. Similar results were obtained with IR phosphotransferase activity. These studies indicate, therefore, that the sensitivity of the IR tyrosine kinase to insulin is increased in most breast cancers and may provide a selective ligand-dependent growth advantage to these tumors (39).

b) Cultured breast cell lines:

125I-Insulin binding studies carried out in intact cells showed that insulin binding was correlated with the IR protein content, as measured by radiomunnoassay. MCF-7 cells had the highest binding capacity (36) while the T47-D cells had the lowest binding capacity. The affinities of both classes of binding sites were relatively higher in ZR-75 cells than in MCF-7.

The insulin-stimulated IR phosphotransferase activity was 5-10 fold higher in all breast cancer cells (except MDA-MB231) in respect to non malignant breast cells. The greatest increase in IR tyrosine kinase activity was observed in ZR-75 cells (Figure 3). No response to insulin was observed in the receptor tyrosine kinase activity of IR extracted from MDA-MB231 cells (40). As it will be described later, the lack of insulin effect in the MDA-MB231 derived IR is due to the presence, in these cells, of a potent tyrosine kinase inhibiting activity. Insulin induced a dose-dependent growth response in all cell lines except MDA-MB-231 cells (36,40). In the responsive cell lines insulin was effective at a concentration of 0.1-1.0 nM. Maximal growth stimulation was observed in the presence of 100 nM insulin (Figure 4). To demonstrate that cell growth was mediated by the IR and not by the related IGF-I-R, we blocked IGF-I-Rs with the monoclonal antibody dIR3. Incubation with dIR3 inhibited approximately 50% of the insulin effect on MCF-7 cells whereas it had no effect on the insulin effect in ZR-75 and T47-D cells. Further, the monoclonal antibody MA-5, an insulin agonist, stimulated cell growth similarly to insulin in ZR-75 and T47-D cells but only approximately one half that of insulin in MCF-7 cells (36). These findings indicated that in breast cancer cells insulin-stimulated cell growth is mediated by IRs. Results obtained in MCF-7 prompted us to study in more detail the characteristics of IRs in this cell line and lead to the discovery of an atypical receptor of the insulin/IGF-I family (see below).

Our in vitro studies on insulin-stimulated breast cancer cell growth confirm earlier studies obtained in
These studies suggest, therefore, that: a) PC-1 is overexpressed in MDA-MB231 cells and is a major contributor to the insulin resistance of this cell line; b) other unidentified factors with tyrosine kinase activity are present in MDA-MB231 cells.

III- Possible mechanisms of insulin mitogenic effect potentiation in breast cancer

a) Insulin/IGF-I hybrid receptors

Hybrid insulin/IGF-I receptors (IR/IGF-I-R hybrids) are heterodimers formed by one IR α and β subunit complex and one IGF-I-R α and β subunit complex (42, 43). IR/IGF-I-R hybrid receptors have been described in IR gene transfected cells and in placenta, where IR expression is high (42, 43). Since both IR and IGF-I-R are usually overexpressed in human breast carcinomas we evaluated whether these hybrid receptors were also present in breast cancer cell lines the proportion of IR/IGF-I-R hybrids ranged from 32 to 86% of total 125I-IGF-I binding receptors and was directly related to the IR level. In the breast cancer tissue specimens examined IR/IGF-I-R hybrids accounted for more than 70% of total 125I-IGF-I binding receptors (unpublished data). Since hybrid IR/IGF-I-R preferentially bind IGF-I (42), IR overexpression in breast cancer cells may contribute to the potent mitogenic effect of IGF-I by increasing the number of hybrid IR/IGF-I-R.

b) Insulin receptor isoforms in breast cancer

The human insulin receptor is expressed in two natural isoforms differing for the absence (IR-A) or the presence (IR-B) of a 12 amino acid sequence corre-
These data suggest that one possible mechanism accounting for a high sensitivity to insulin of certain breast cancers is the relative prevalence of the IR-A isoform.

c) Atypical IGF-I like receptors.
In MCF-7 cells, in addition to the typical IRs and IGF-I-Rs Milazzo et al. (44) found an atypical IGF-I like receptor that binds both insulin and IGF-I with high affinity. Preliminary data suggest that also some breast cancer specimens may contain this form of atypical receptors. These findings suggest that insulin, especially in hyperinsulinemic patients, may stimulate cancer cells via atypical IGF-I-R.

TRANSFORMING POTENTIAL OF INSULIN RECEPTOR

Overexpression of tyrosine kinase receptors including the EGF receptor, the Met/HGF receptor and the IGF-I receptor are able to induce a ligand-dependent transformed phenotype and, therefore, play a role in cancer initiation and/or progression (45, 47).

We studied NIH3T3 cells transfected with the human insulin receptor cDNA (3T3/HIR) and overexpressing the IR protein at levels comparable to those found in over 10% human breast cancers (48). When stimulated with insulin, these cells lost contact inhibition and formed large multilayered focal aggregates when cultured in petri dishes and colonies in soft agar (Figure 6). These phenotypic changes, characteristic of cell transformation, were reversed when the IR was blocked with MA-10, a specific monoclonal antibody with blocking activity of the IR. IGF-I was unable to mediate these phenotypic changes (48).

Similar results were obtained by Frittitta et al. (49) by transfection of IR cDNA in the immortalized non malignant human breast epithelial cells, 184B5, indicating that also in human breast cells IR overexpression plays a role in the appearance of a ligand-dependent phenotype. Neither 3T3/HIR nor IR transfected 184B5 cells were able to form tumors when transplanted in nude mice. These findings suggest that, in addition to the insulin receptor overexpression, other factors may be necessary for full malignant transformation.

In recent years Baserga et al. have gathered evidences suggesting that IGF-I receptor has a permissive role in cell transformation (50). These studies originated from the basic observation that embryonic fibroblasts from IGF-I receptor deficient mice (R- cells) are unable to undergo transformation after being transfected with SV-40 Large T anti-
gene DNA (51). This group has then extended this observation by studying different model systems. C6 rat glioblastoma cells were rendered IGF-I receptor deficient by the use of antisense RNA (52); these cells, unlike wild type parental cells, were unable to form tumors when transplanted into syngeneic rats. These studies provide evidences that IGF-I-R has a special relevance in establishing and maintaining the transformed phenotype and may represent a target of choice for future therapeutic approaches. Given the similarity between IR and IGF-I-R and the ability of these molecules to recombine forming hybrid insulin/IGF-I receptors it is possible to hypothesize a role of IR similar to that of IGF-I-R in maintaining cell transformation (50).

POSSIBLE ROLE OF INSULIN AND INSULIN RECEPTORS IN OTHER MALIGNANCIES

Insulin has been shown to stimulate mitogenesis in a variety of malignant cell lines. The role of insulin and IR in malignancies other than breast cancer is, however, less defined since specific studies are not available. However, a body of evidence links insulin resistance and hyperinsulinemia to the development of colon cancer (53). Epidemiological evidences have related the development of colon cancer to central obesity, physical inactivity, a diet low in fruits and vegetable and high in red meat and processed foods. All these risk factors are major determinants of insulin resistance and/or high glycemic load and hyperinsulinemia. IRs and IGF-I-Rs are present in most normal and neoplastic hemopoietic cells. IRs have been found preferentially expressed in B-lymphoblasts, T-lymphocytes, plasmacytoma cells. Pileme et al. (54) have described a murine lymphoid T-cell leukemia which is dependent on insulin for growth and which does not respond to IGF-I. Clearly, further work is necessary to establish the contribution of insulin to the proliferation and differentiation of neoplastic hematopoietic disorders.

Recently, we found that IRs were expressed at high levels in normal and neoplastic human thyroid cells. Further, in most differentiated thyroid carcinomas IRs levels were higher than the mean value + 2SD in normal tissue (unpublished data). The potential role of IRs in thyroid cancer also deserves more work.

CONCLUSIONS

Epidemiological evidences indicate that central obesity is related to an increased risk of breast cancer and to an increased cancer aggressiveness. We have observed that the large majority of human breast carcinomas have increased levels of functional IR which are mainly expressed by the malignant epithelial cells. These findings raise the possibility that insulin receptor overexpression may confer selective growth advantage to breast cancer cells, especially in clinical conditions associated with hyperinsulinemia. IR measurement in breast carcinomas may have an important prognostic impact by predicting patients with node-negative carcinomas which will relapse and which, therefore, would benefit from adjuvant chemotherapy.

IR overexpression has also been shown to induce ligand-dependent cell transformation, like other tyrosine kinase receptors. Further work may provide evidence that IRs, like IGF-I-Rs, have a broad role in cancer in establishing and maintaining the transformed phenotype. Hyperinsulinemia and overexpression of IRs by neoplastic cells are increasingly recognized to play a role in malignancies other than breast cancer. These findings open new possibilities for innovative therapeutic strategies. The identification of an endogenous IR tyrosine kinase inhibiting activity may be a step forward in the design of these therapies.

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